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# Growth Enhancement of *Spirulina platensis* through Optimization of Media and Nitrogen Sources

**Mostafa M. El-Sheekh[(1)#], Lamiaa H.S. Hassan[(2)], Hanaa H. Morsi[(2)]**
*[(1)]Botany Department, Faculty of Science, Tanta University, Tanta, Egypt; [(2)]Botany and Microbiology Department, Faculty of Science, Menoufia University, Egypt.*

*SPIRULINA* has drawn attention throughout the last decades. It is an essential source of valuable products, such as proteins, phycobilins, carotenoids, phenolics, and unsaturated fatty acids. These products had been used in medicine, pharmaceutical, and agriculture. In this study, the effect of different growth media on *Spirulina platensis* was studied after the cultivation at optimum growth conditions; continuous light intensity (60µmol photons $m^{-2}s^{-1}$), temperature (25±3ºC), and pH (9.0 ± 0.2). The growth was estimated through 42 days by optical density (OD), cell counting (CC), and chlorophyll contents. The results showed that Kuhl's medium was the optimum for *S. platensis* with the highest results, i.e.: (OD), (CC) and Chlorophyll content increased 11.57 times, 19.55 times, and 22.66 times of the initial record, respectively. $KNO_3$ showed the best nitrogen source for *S. platensis*, where the different parameters of growth elevated to 3.56 times OD, 7.33 times CC, and 1.91 total chlorophyll more than their corresponding control.

**Keywords:** Biomass, Cyanobacteria, Growth media, Growth optimization, Nitrogen source, *Spirulina*.

## Introduction

*Spirulina* species are non-toxic aquatic filamentous blue-green algae, which may habitat in marine, freshwater and alkaline lakes. In 1981, the FDA (Food Drug Administration) approved *Spirulina* by the issuance of a GRAS (generally recognized as safe) certificate, so *Spirulina* is legally considered as a food or food supplement without any risk to human health (Verne`s et al., 2019). It has nutraceutical benefits, as it is a source of rich nutritional compounds, such as proteins, essential amino acids, carbohydrates, vitamins, nicotinate, biotin, folic acid, minerals, phenolics, pigments (chlorophyll, carotenoids, c-phycocyanin, and phycobilins), poly-unsaturated fatty acids and pantothenic acid (Shao et al., 2019; Mohy El-Din, 2020). Its valuable products set *Spirulina* as the most cultivated microalga worldwide (Ghaeni et al., 2015; Matos et al., 2017; Shao et al., 2019). These compounds increase their biological function and commercial uses (Chandi & Gill, 2011; Rizzo et al., 2015; Dubini & Antal, 2015). Various industries use *Spirulina*; in cosmetics, pharmaceutical products, poultry industries, biofertilizers, food and feed, and plant biostimulants (Priyadarshani & Rath, 2012; El-Sheekh et al. 2014a; Suganya et al., 2016; Soni et al., 2019). Ayehunie et al. (1998) reported that *Spirulina platensis* produced sulfated polysaccharide calcium spirulan with the antiviral activity, which inhibits the entry of enveloped viruses to the cell (measles virus, Herpes simplex, and cyto-Megalo viruses). *Spirulina* sp. exhibited antimicrobial activities against various bacterial and fungal pathogens (El-Sheekh et al. 2014b; Mohy El-Din, 2020). *Spirulina* can be used in environmental applications, such as nutrient removal, as compared with *Chlorella vulgaris* (El-Naggar & El-Sheekh, 1998; Markou, 2015). It was reported that *Spirulina* eliminated 99% of phosphate from the wastewater (Phang et al., 2000). It can produce antioxidant compounds that, it prevents some diseases, improves the immune system, and protects from oxidative stress (Barrow & Shahidi, 2007). It enhances lactic acid

#Corresponding author email: mostafaelsheikh@science.tanta.edu.eg
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bacteria in the gastrointestinal tract that leads to improving body hormones. *Spirulina* is also used for the treatment of other diseases like arthritis, anemia, cardiovascular diseases, diabetes, and cancer (Mani et al., 2007; Rizwana et al., 2018). *Spirulina* is characterized by some physical and chemical factors that affect biomass production. Some factors influence the growth, such as suitable alkaline pH value, $CO_2$ concentration, available salts, nutrients, temperature, and light intensities (Sudhakar & Premalatha, 2015; Soni et al., 2016). *Spirulina* also requires a relatively alkaline pH. In order to maintain high alkaline pH, high amounts of sodium salts must always be there in the culture medium (Richmond & Grobbelaar, 1986; Vonshak, & Richmond, 1988). The availability of nutrients, especially nitrogen and carbon, affects the cell growth and biochemical composition of microalgal cells (El-Sheekh et al., 2010, 2012). The growth and cell biomass are regulated by nitrogen uptake, as nitrogen is an essential component for amino acids and protein production (Chow, 2012). The nitrate is a common nitrogen source in microalgae culture. The c-phycocyanin content in *Spirulina platensis* effects by nutrient contents in the culture media, especially nitrate content, which could lead to the reduction or inhibition of c-phycocyanin, (Manirafasha et al., 2018; Mohy El-Din, 2020).

This study aims to elevate the biomass production of *Spirulina platensis* by using optimizing growth media under the optimum growth conditions.

## Materials and Methods

*Algal strain*

*Spirulina platensis* was isolated from freshwater canals in Shebin El-kom, Menoufia Government, Egypt. The microalga was identified up to species under an inverted divert light microscope following the keys of (Bold & Wynne, 1978; Prescott, 1984; Yamagishi, 1992; Vymazal, 1995). The width of the trichomes, composed of cylindrical shorter than broad cells, a diameter of 8 to 10µm and length of tens to hundreds of µm the coiled with a diameter close to 5-6µm.

*Growth media*

A comparison study was done between different media to optimize the growth of *S. platensis*. These were carried out according to Sharma et al. (2012) and Abou-El-Souod et al. (2016). Cultures were subjected to five different media of different chemical compositions and pH: BG-11 Medium (Ilavarasi et al., 2011), Bold's basal medium (Bischoff & Bold, 1963), Zarrouk's medium (Zarrouk, 1966), Modified Chu's-10 medium (Stein, 1973) and Kuhl's medium (Kuhl & Lorenzen, 1964), as described in Table 1.

*Experimental setup*

*Different media optimization*

Five conical flasks (250mL) were prepared, each one containing 90mL of each medium (Table 1), and inoculated uniformly with 10% (v/ v) *S. platensis* subculture. The culture flasks were aerated by air pumps and incubated at 25±3ºC under continuous illumination provided from three standard cool white LED lamps (9W) 60µmol photons $m^{-2}s^{-1}$. The experiment for each medium was carried out in three replicates (Joshi et al., 2018). The pH was adjusted to be suitable for this *S. platensis* growth (9.0±0.2), using 1M sodium hydroxide (NaOH) and 1N hydrochloric acid (HCl) by using pH meter (SCHOTT CG 843).

*Effects of nitrogen source*

Different nitrogen sources such as $Ca(NO_3)_2$, $NaNO_3$, $(NH_4)_2SO_4$, $NH_4NO_3$, and urea [$CO.(NH_2)_2$] were tested on Kuhl's medium for *S. platensis*. Culture flasks were sterilized in an autoclave at 121°C for 20min (Abou-El-Souod et al., 2016; Salunke et al., 2016). The experiment was cultivated as the previous conditions.

*Growth analysis*

According to Sharma et al. (2012) and Abou-El-Souod et al. (2016), the growth curves were observed and followed at week intervals using: i) optical density at 680nm, ii) Cell counting and (as a unit or filament) iii) Chlorophyll contents. The optical density depends on using a colorimetric spectrophotometer at 680nm (Spectro UV-VIS DUAL BEAM 8 AUTO CELL UVS-2700). While cell count examination was performed using a hemocytometer (Neuberger improved), according to Gerloff et al. (1950). The total chlorophyll contents were detected by using the methanol extraction method of Mackinney (1941) and measuring the wavelength on (Spectro UV-VIS DUAL BEAM 8 AUTO CELL UVS-2700).

**TABLE 1. Composition of all described media g/L**

| Chemicals | BG-11 | Bold's basal | Zarrouk's | Modified CHU's-10 | Kuhl's |
|---|---|---|---|---|---|
| $NaNO_3$ | 1.500 | 0.250 | 2.500 | - | - |
| $KNO_3$ | - | - | - | - | 1.011 |
| $Ca(NO_3)_2$ | - | - | - | 0.232 | - |
| $K_2HPO_4$ | 0.040 | 0.075 | 0.500 | 0.010 | - |
| $KH_2PO_4$ | - | 0.175 | - | - | - |
| $Na_2HPO_4$ | - | - | - | - | 0.089 |
| $NaH_2PO_4$ | - | - | - | - | 0.621 |
| $MgSO_4.7H_2O$ | 0.075 | 0.075 | 0.200 | 0.025 | 0.2465 |
| NaCl | - | 0.025 | 1.000 | - | - |
| $CaCl_2.2H_2O$ | 0.036 | 0.025 | 0.040 | - | 0.0147 |
| Citric acid | 0.006 | - | - | 0.0035 | - |
| Ferric citrate | 0.006 | - | - | 0.0035 | - |
| $FeSO_4.7H_2O$ | - | 1mL Stock* | 0.010 | - | 0.690*** |
| EDTA (disodium salt) | 0.001 | 1mL Stock** | 0.080 | 1.000 | 0.930*** |
| $NaHCO_3$ | - | - | 16.800 | - | - |
| $Na_2CO_3$ | 0.020 | - | - | 0.020 | - |
| $K_2SO_4$ | - | - | 1.000 | - | - |
| $Na_2SiO_3.9H_2O$ | - | - | - | 0.044 | - |
| Trace metal A5 | 1.0mL | 1.0mL | 1.0mL | 1.0mL | 1.0mL |
| $H_3BO_3$ | 2.86 | 11.400 | 2.860 | 2.400 | 0.061 |
| $MnCl_2.4H_2O$ | 1.810 | 1.44 | 1.810 | 1.400 | 0.169 |
| $ZnSO_4.7H_2O$ | 0.222 | 8.82 | 0.222 | 0.222 | 0.287 |
| $Na_2MoO_4.2H_2O$ | 0.390 | 0.71 | 0.017 | 0.390 | 0.390 |
| $CuSO_4.5H_2O$ | 0.079 | 1.570 | 0.079 | 0.079 | 0.0025 |
| $Co(NO_3)_2.6H_2O$ | 0.049 | 0.490 | - | 0.049 | 0.0005 |
| Distilled water | 1.0L | 1.0L | 1.0L | 1.0L | 1.0L |
| pH | 7.5±0.2 | 6.8±0.2 | 9.0±0.2 | 8.5 for cyanobacteria | 6.8±0.2 |

*EDTA stock is prepared for Bold's Basal medium by dissolving 5.0 g EDTA (disodium salt) to 3.1 g KOH in 100mL distilled water.
**Iron solution consists of 4.98 g $FeSO_4.7H_2O$, 1.0mL concentrated $H_2SO_4$ in 1 Liter of distilled water.
*** In Kuhl's medium Fe-EDTA complex: dissolving of 0.69 g $FeSO_4.7H_2O$ and 0.93 g disodium-salt of EDTA in 80ml of distilled water by boiling for a short time. After cooling to room temperature it is made up to 100mL.

*Growth curve*

Finally, *S. platensis* growth curve was tested under the previous optimum conditions, using Kuhl's medium at pH (9±0.2), $KNO_3$ as a nitrogen source, and artificial illumination using three white Led lamps (60µmol photons $m^{-2}s^{-1}$). The growth was incubated at 25±2°C. At the optimum growth, total chlorophyll, carbohydrates (Dubois et al., 1956), protein (Lowry et al., 1951), and lipid (Ahlgren & Merino, 1991) were estimated.

*Statistical analysis*

The obtained data were performed at three replicates (n= 3). The variance and statistical significance were established with ANOVA test at 0.05 level using SPSS for Windows (SPSS 16). The results were presented as mean ± standard deviation (SD). Least significant difference (LSD) at 5% probability ($P \leq 0.05$).

## Results

*Different media optimization*

Figures 1 and 2 showed that the optimum growth was achieved on 35$^{th}$ day, when the optical density (OD) and cell count (CC) increased 11.57 times and 19.55 times of the initial record, respectively. Zarrouk's medium

was recorded by 5.49 times in OD and 12.03 times in CC. Bold's basal medium was recorded 4.93 times in OD and 11.53 times in CC. In comparison, BG-11 medium OD and CC were increased 2.91 and 6.02 times of the initial record. There was no growth at modified CHU's-10, unfortunately, all the cells gradually dead.

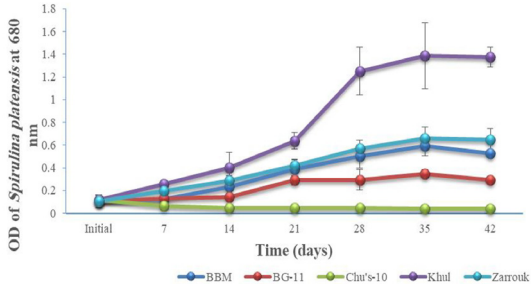


**Fig. 1. Effect of different media on the growth of *Spirulina platensis* as (OD)**

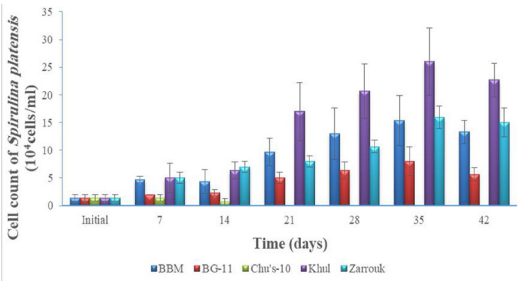


**Fig. 2. Effect of different media on the growth of *Spirulina platensis* as (CC)**

Figure 3 showed the estimation of total chlorophyll contents in the cultivated *S. platensis* at the best medium for getting the maximum biomass. Kuhl's medium also showed the optimum Chlorophyll content 22.66 times of the initial record, on the 35th day of cultivation. Zarrouk's medium increased the Chlorophyll content by 15.27. In the case of Bold's basal medium and BG-11 medium, they were increased by 13.45 times and 12.09, respectively. CHU's-10 showed no growth, and Chlorophyll decreased.

*Effect of different nitrogen sources*

The results in Figs. 4, 5, and 6 showed that $KNO_3$ gave the highest growth of *S. platensis* 3.56 fold (OD), 7.33 fold (CC), and 1.91 fold total chlorophyll at the 28th day of the growth. OD of $Ca(NO_3)_2$ increased by 2.59 fold, 5.22 fold (CC), and 2.05 fold in total chlorophyll. While $NH_4NO_3$, $(NH_4)_2SO_4$, and urea showed declined the *S. platensis* growth, until the whole cell death.

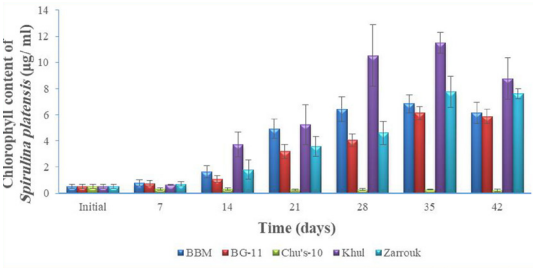


**Fig. 3. Effect of different media on the growth of *Spirulina platensis* as (Chl. content)**

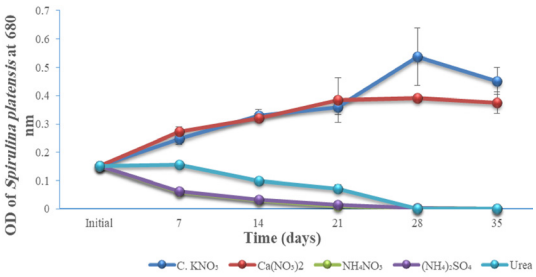


**Fig.4. Effect of different Nitrogen sources in Kuhl's medium on growth (OD) of *Spirulina platensis***

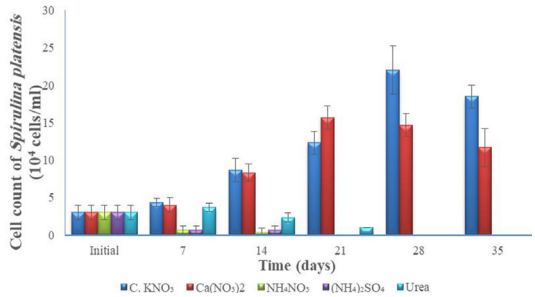


**Fig.5. Effect of different Nitrogen sources of Kuhl's medium on growth cell count (CC) $10^4$ cells/ mL of *Spirulina platensis***

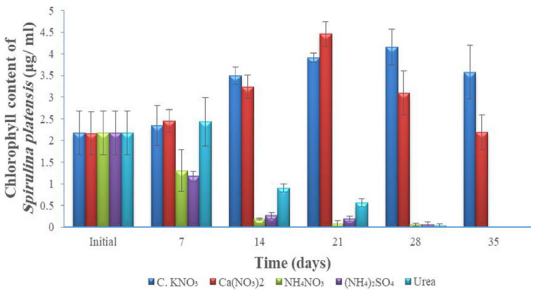


**Fig.6. Effect of different Nitrogen sources of Kuhl's medium on total chlorophyll content of *Spirulina platensis***

*Spirulina platensis growth curve*

*S. platensis* growth curve on Kuhl's medium, using $KNO_3$ as a nitrogen source, was detected by OD (Fig. 7). The optimum growth level of *S. platensis* was achieved on the 28th day of the growth. The total chlorophyll content (5.21µg.

$mL^{-1}$), carbohydrates (35.10mg.$L^{-1}$), protein (23.44mg.$L^{-1}$), lipids (600mg. $L^{-1}$).

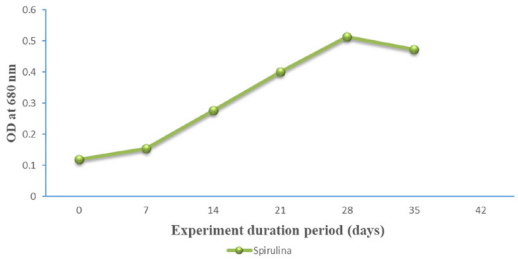


**Fig. 7. Growth curve of *Spirulina platensis* at the optimum growth conditions**

## Discussion

The results indicated that Kuhl's medium was the best medium for *Spirulina platensis* cultivation. However, Kuhl's medium is widely used for the cultivation of green microalgae, and it gave the maximum growth rate of *S. platensis* during this study. Abou-Gabal et al. (2018) studied the optimization of *S. platensis* using Kuhl's medium, but their study showed that Kuhl's medium was not the optimized growth medium for *S. platensis* cultivation. However, during previous studies, Zarrouk's medium was the most popular medium used for *Spirulina* cultivation (Abd El-Monem et al., 2019); it gave a lower growth rate than that was achieved from Kuhl's medium. Joshi et al. (2018) reported that BG-11 growth medium was the best for *Spirulina platensis*, when they tested three different media i.e: BG- 11, CHU, and Walne.

The isolated *S. platensis* was sampled from freshwater canals, which contain fewer carbonate concentrations comparing to the marine water. Changes in microalgae culture parameters can affect biomass growth, modifying the structure and biochemical contents (nutrients, vitamins, enzymes, and toxins) (Xie et al., 2017; Piazzi et al., 2019; Mohy El-Din, 2020). Another expectation was nitrogen (concentration of nitrogen and its source), as (Soletto et al., 2005; Çelekli & Yavuzatmaca, 2009), reported that nitrogen concentration and nitrogen source had a great effect on the growth of *Spirulina*. Kavita & Mohanty (2000) reported that when *S. platensis* was exposed to high sodium concentration, a 30% increase in intracellular sodium accumulation and caused a little change in dry mass and chlorophyll content. *S. platensis* requires relatively high pH, which inhibits the contamination by other microalgae and elevated chlorophyll production (Richmond & Grobbelaar, 1986; Hu, 2004). Vincent & Silvester (1979) reported that the pH had an effect on algal physiology and nutrient availability. As the pH-controlled the solubility of carbon source and minerals in the culture. He also noted that *Spirulina* best growth rate was achieved at pH values between 9 and 11.

Nitrogen is an essential nutrient for the microalgal biomass (Li et al., 2018), *S. platensis* can assimilate various nitrogen sources. Although ammonium is the preferred nitrogen source for *Spirulina* sp., it is toxic to cells at elevated concentrations and can inhibit the growth and cause cell death. Salunke et al. (2016), regarded that urea is more effective than $KNO_3$ for *S. platensis*. The previous studies demonstrated that the availability and concentration of nitrogen source influence the microalgal proteins content (Markou, 2015; Li et al., 2016)

Delrue et al. (2017) reported that when *Spirulina* was cultivated for 40 days cultivation in a 1000L- photobioreactor during spring 2015. This period of cultivation helped to increase the possibility of *Spirulina* growth in the photobioreactor and improving biomass productivity. In addition to this, the initial density of the inoculum sample can affect the growth period. As shown in Van-Khanh et al. (2017) study, the lowest initial density of *Spirulina* inoculum took long cultivation time more than that of the highest initial density to reach the maximum growth density.

## Conclusion

The presented results showed that *Spirulina platensis* biomass could be significantly increased by using Kuhl's medium. The optimum value of media factors like pH, nitrogen source, phosphate source, and salinity can enhance the growth rate and biomass. It is concluded that the modification of Kuhl's medium by elevating the pH value (9.0±0.2), and using $KNO_3$ as a nitrogen source, allow the improvement of *S. platensis* biomass production.

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*Conflict of interest:* The authors declare that there is no conflict of interest.

*Author contribution statements:* M.E. and H.M. supervised the work and conceived the original idea. M.E. finalized the manuscript. L.H. carried out the experiments, wrote the draft of the manuscript with support from M.E. and H.M.

*Ethical approval:* Not applicable

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## تعزيز نمو *Spirulina platensis* من خلال وسائط النمو الأمثل ، ومصادر النيتروجين المختلفة

**مصطفى محمد الشيخ[1]، هناء حسنين مرسي[2]، لمياء حسن شحاته حسن[2]**

[1]قسم النبات - كلية العلوم- جامعة طنطا -طنطا - مصر،[2] قسم النبات و الميكروبيولوجي- كلية العلوم- جامعة المنوفية- المنوفية- مصر.

لقد لفتت طحلب سبيرولينا الانتباه طوال العقود الماضية. فهي مصدر في غاية الأهمية للمنتجات النافعة، مثل البروتينات والفيتوبيلين والكاروتينات والفينولات والأحماض الدهنية غير المشبعة. وقد تم استخدام هذه المنتجات في الطب والصيدلة والزراعة. خلال هذه الدراسة تم اختبار تأثير أوساط النمو المختلفة على *Spirulina platensis* بعد الزراعة في ظروف النمو المثلى؛ الضوء المستمر تصل شدته إلى (60 ميكرون فوتونات.م$^{-2}$. ثانية$^{-1}$)، درجة الحرارة (25 ± 3 $^{0}$س)، ودرجة الحموضة تصل إلى (9.0 ± 0.2). و تم تقدير النمو خلال 42 يومًا من خلال قياس الكثافة البصرية (OD)، وعدد الخلايا (CC) ومحتوى الكلوروفيل. أظهرت النتائج أن الوسط الغذائي Kuhl هو الأمثل لـنمو *S. platensis* مع أعلى النتائج، أي: (OD) و (CC) و محتوى الكلوروفيل زاد 11.57 ضعف و 19.55 ضعف و 22.66 ضعف من القياسات الأولي، على التوالي. كما أظهر $KNO_3$ أفضل مصدر للنيتروجين لنمو *S. platensis* الذي تم تقديره كـ 3.56 ضعف من OD و 7.33 ضعف من CC و كذلك 1.91 ضعف من الكلوروفيل.